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The infant human thymus was analyzed in a search for early precursor cells that might be targets for HIV infection. The more mature cells were removed by depletion procedures and early cells isolated by multiparameter fluorescence activated cell sorting. The earliest T-lineage cells obtained were $CD34^{+}7^{+}2^{+}1^{-}3^{-}4^{-}8^{-}$, in contrast to mouse thymus where the earliest cells expressed CD4. However, human thymic dendritic cells (DC) were isolated and these expressed high levels of CD4. Their surface phenotype was $CD1^{-}2^{-}3^{-}4^{+}8^{-}7^{-}34^{-}44^{+}$ MHC class I^{+} class II^{+}. These DC were tested for infectability with HIV-1, by pulsed contact with virus, culture for 2-7 days, then PCR for gag or immunoperoxidase staining for p24. A high proportion of thymic DC showed productive infection with virus. However, infection was restricted to the macrophage trophic HIV strains, lymphotrophic isolates being non-infective. The results have implications for the early stages of AIDS development.

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APPENDIX:

a. Published paper: Sotzik F, Boyd A, Shortman K.
Surface antigens of human thymocyte populations defined by CD3, CD4 and CD8 expression: CD1a is expressed by mature thymocytes but not peripheral T cells. Immunol Letters 36: 101-106, 1993

b. Published paper: Sotzik F, Rosenberg Y, Boyd AW, Honeyman M, Metcalf D, Scollay R, Wu L, Shortman K.
Assessment of CD4 expression by early T-precursor cells and by dendritic cells in the human thymus.
J Immunol 152: 3370-3377, 1994

c. Published paper: Winkel K, Sotzik F, Vremec D, Cameron PU, Shortman K.
CD4 and CD8 expression by human and mouse thymic dendritic cells.
Immunol Lett 40: 93-99, 1994

d. Data demonstrating the infection of dendritic cells with HIV-1.

INTRODUCTION

This research programme was initiated following our finding (1, 2) that in the adult mouse thymus the very rare, earliest T precursor cells expressed, very surprisingly, a moderate level of the mature T-cell marker CD4. Since CD4 is known to be the main receptor for HIV binding and entry into cells (3, 4) it seemed that, if such an early precursor in the human thymus was $CD4^+$, it could be a target for HIV-1. This would have significant consequences for the development of T cells in an infected individual.

Accordingly, we set out to look for an equivalent very early T precursor in the human thymus. Although we were successful in isolating putative very early human thymus T precursors, these cells were clearly $CD4^-$, in contrast to the mouse thymus. However, we did note in our analyses a quite distinct minor group of cells in the human thymus that expressed high levels of CD4. These were not T-lineage cells and their nature was initially a puzzle; we eventually identified them as human thymic dendritic cells (DC), because of our ongoing and quite separate work on mouse thymus DC (5, 6). Although they resembled the murine DC in many respects, we were able to document another marked species difference. Human thymic DC express high levels of CD4, whereas mouse thymic DC express high levels of CD8.

These findings led to a change of focus from our original grant proposal, since it was the human thymic DC, not the early T precursors, which now seemed likely to be HIV-1 targets. Accordingly we formed a collaboration with Dr. Paul Cameron of the Macfarlane Burnet Centre, Melbourne, who had considerable experience with the interaction of HIV with DC in the laboratory of R. Steinman, The Rockefeller University, NY. In this collaborative study we found that the human thymic DC are indeed good targets for HIV, although there is a strong bias to infection with particular HIV isolates.

CHARACTERISTICS OF HUMAN THYMOCYTES

The work in this section has been published in full detail and a reprint is included as Appendix (a). This section is therefore only a general commentary.

Murine thymocytes have generally been analyzed in greater detail, with more surface markers, than human thymocytes. This was certainly the situation in this laboratory where our experience had largely been confined to the mouse thymus. Accordingly, we first obtained a large repertoire of potentially useful monoclonal antibodies (mAb) specific for various human T-cell and other lineage markers (finally 23 in all), and rather than purchasing them grew the hybridomas ourselves, purified the mAb and conjugated each of them to 2 different fluorochromes. Each mAb was titrated, both for cytotoxicity and fluorescence, on the appropriate target cell. Armed with this repertoire, we carried out a basic characterisation of human thymocyte suspensions, using fragments of infant human thymus removed during routine thoracic surgery.

Most of our three-colour fluorescence analyses of the human thymus gave results in accordance with published studies, and with results on the mouse thymus. However, we observed three notable features that were new, namely:

1. Mature, medullary "single positive" ($CD4^-8^+$ and $CD4^+8^-$) thymocytes expressed CD1a whereas peripheral T cells did not. This was in contrast to most published work where it had been apparently assumed that mature thymocytes were $CD1a^-$ (7). The loss of CD1a therefore marks some post-thymic developmental step, not positive selection itself.

2. A small subgroup of peripheral T cells are $CD7^-$; the significance of these is uncertain.

3. Human thymus contains $CD4^+8^-3^-$ "immature single positives" (a step between $CD4^-8^-$ and $CD4^+8^+$ thymocytes) but no $CD4^-8^+3^-$ immature single positives which are a major intermediate in the mouse thymus; human thymocytes thus acquire CD4 before CD8 during this stage of development.

EARLY HUMAN THYMIC T PRECURSORS

The work in this section has been published in full detail and a reprint is included as Appendix (b). This section is therefore only a general commentary.

To study the earliest precursors in the thymus, <0.1% of all thymocytes, depletion procedures are first used to remove the bulk of more mature cells. We adopted a two-step procedure. The first used cytotoxic mAb and complement treatment, less efficient with human than with mouse cells. Accordingly, only mAb giving good killing were used. Anti-CD3 was avoided since this is known to cap-off CD3 and render some cells resistant to killing. After removal of dead cells, the recovered viable cells were again depleted using a different series of mAb, followed by anti-mouse Ig coated magnetic beads and removal of coated cells with a magnet. At this stage mAb recognizing non-T lineage cells were included in the depletion cocktail, to remove minor populations which would contaminate the precursor cells. At this stage, when <0.6% of thymocytes remained, the recovered cells were labelled in 2-3 fluorescent colours with mAb and analyzed for the expected markers of very early T precursor cells.

Our initial depletions used anti-CD8, anti-CD3, and anti-CD2, since we initially followed models suggesting the earliest T lineage thymocytes lacked any CD2 (7). This depletion gave us a preparation that included dendritic cells (see later) but few early T cells. We found, in agreement with Terstappen (8), that early T-lineage thymocytes expressed some CD2. We then modified our procedure, adding anti-class II MHC to remove dendritic cells and using anti-CD1a instead of anti-CD2 to deplete later T-lineage cells. This approach gave us a suitable preparation to analyze further.

The depletion procedure still left a few probable later T-lineage cells (perhaps with markers capped off) but these were readily recognized as cells expressing low levels of class I MHC. We were able to distinguish putative early T-lineage cells by analogy with the cells we purified from the mouse thymus (1, 2) and based on the studies of others on early T cells and T precursors in human thymus and bone marrow (7, 8). The surface phenotype of these cells was: $CD34^+$ $CD7^+$ $CD44^+$ Class I MHC^{high} and $CD1a^-$ $CD8^-$ $CD3^-$. However, none of these cells expressed any detectable CD4. Thus the situation differed from the mouse thymus, and there was no strong case for believing these earliest thymocytes would be especially sensitive to infection with HIV.

HUMAN THYMIC DENDRITIC CELLS

The work in this section has been published in full detail and reprints are included as Appendix (b) and Appendix (c). This section is therefore only a general commentary.

Human thymic dendritic cells (DC) had previously not been studied thoroughly, and most of the previous characterization (claiming they were $CD4^-$ and $CD1a^-$) proved incorrect. We initially detected them as a puzzling $CD4^+$ population in our depleted, supposedly early T-precursor enriched, preparations. However, they were not T-lineage cells because they were too large and lacked CD7; they were not early precursor cells since they lacked CD34 and produced no colonies in agar; they showed none of the markers, surface or cytoplasmic, of NK cells, B cells, macrophages, monocytes or granulocytes. Their surface phenotype was: $CD1a^-2^-3^-4^{++}8^-7^-34^-44^+11c^+14^-16^-19^-56^-$ class I MHC^{++} class II MHC^+.

The high expression of class I and class II MHC and of CD11c suggested they were DC. Although they had rounded up during isolation, incubation in simple media revealed a characteristic DC morphology, with convoluted shape and dendritic extensions. Finally, they were very effective stimulators in a mixed-lymphocyte reaction, the hallmark of DC.

Having characterized these thymic DC, we then improved the procedure for their isolation, modifying a method we had already developed for murine DC (5). This involved collagenase digestion of thymus fragments, EDTA-chelation of divalent metals to release DC from complexes with thymocytes, selection of light density cells, depletion of non-DC with mAb and immunomagnetic beads, then sorting for cells expressing very high levels of class I MHC. This produced a good yield (for DC!) of 1 DC per 10^3 thymocytes.

A startling species difference emerged from a direct comparison of mouse and human DC isolated by equivalent procedures. Human thymic DC are $CD4^+8^-$, whereas murine thymic DC are $CD4^-8^+$. The basis of this remains unclear. However, it served to emphasize the possible importance of CD4 on human thymic DC as a possible ligand allowing HIV-1 infection.

INFECTION OF THYMIC DENDRITIC CELLS WITH HIV-1

The work in this section was in collaboration with P. Cameron at the Macfarlane Burnet Centre, Melbourne. Some of the final results of these studies are still being assayed. The work is currently being written up for submission to J. Exp. Med. An example of the results of this study is given as Appendix (d).

The basic approach was to isolate DC from infant human thymus at the Hall Institute, using the procedure described in Appendix (c), including sorting for cells expressing high levels of class II MHC, to ensure high purity. Control experiments were run to ensure no lymphocyte or monocyte contaminants could contribute to the results. The cells were then transferred to the Macfarlane Burnet Centre (20 minutes distant) for studies on infectivity with HIV-1 in culture.

Around 10^5 pure DC were pulsed for 1.5 hr at 37^o with various HIV-1 strains, washed, then cultured a further 2-5 days. The virus stocks had been pretreated with DNAse I and filtered before use. Controls included DC pulsed at 4^c (no infection) and cell lines permissive or non-permissive for the virus strain used.

For PCR assays cells were washed, lysed, heat inactivated and amplified using gag-specific primers. For histochemical assays cells were washed, cytospin preparations made, fixed with methanol/acetone then stained with p24 specific antibody using horseradish peroxidase-coupled antibody.

The results (see example in Appendix (d)) showed clear expression of gag, and around 40% of the DC were clearly p24 positive, indicators of productive HIV-1 infection. However, this was restricted to macrophage-trophic isolates (such as Ba-L), lymphotrophic isolates (such as IIIb) not giving significant readout. These results have now been confirmed comparing recent patient isolates: 676, a macrophage-trophic patient isolate gave even clearer p24 production whereas 228, a lymphotrophic patient isolate gave negligible p24 production.

In recent experiments transfer of HIV-1 infection to T cells from pulsed and cultured DC has been shown using the Ba-L HIV-isolate, but not using the 111b isolate.

Overall thymic DC are readily infected with "macrophage-trophic" but not with "lymphotrophic" HIV-1 isolates. This marked selectivity of certain HIV-1 isolates for DC had not been reported previously. The results also help resolve an earlier controversy about the ability of HIV-1 to produce a true productive infection of DC, as opposed to simple virus uptake and transfer to T cells. Those DC expressing CD4, such as those we have isolated from the thymus, are subject to a true productive infection by the virus.

CONCLUSIONS

1. Early T-precursor cells, $CD34^{+}$ $CD44^{+}7^{+}2^{+}1^{-}3^{-}4^{-}8^{-}$, may be isolated from the human thymus. In contrast to the murine thymus, these earliest T-lineage cells lack detectable CD4 expression.

2. Thymic dendritic cells, $CD34^{-}44^{+}7^{-}2^{-}1^{-}3^{-}4^{-}8^{-}11c^{+}$, and class I and class II MHC^{high}, may be isolated from the human thymus by the new procedures we have developed. In contrast to murine thymic DC ($CD8^{+}4^{-}$) these cells are $CD8^{-}$ but express high levels of CD4.

3. Human thymic dendritic cells are very sensitive to infection by the macrophage-trophic, but not the lymphotrophic, strains of HIV-1. A productive infection is obtained, which may be transferred to T cells.

4. Future work should examine the influence of such HIV-1 infection of DC on the nature of signals transmitted to T cells. Are DC eliminated by infection? How efficient is the transfer to T cells? Do infected DC still stimulate T cells, or do they cause CD4 T-cell death?

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Surface antigens of human thymocyte populations defined by CD3, CD4 and CD8 expression: CD1a is expressed by mature thymocytes but not peripheral T cells

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(Received 25 January 1993; revision received 2 March 1993; accepted 3 March 1993)

1. Summary

Three colour flow cytometric analysis has been used to analyze the expression of a series of surface antigens on human thymic and peripheral T-cell populations. CD4, CD8 and CD3 were used to divide the populations into the conventional major categories, and the distribution of CD1a, CD2, CD7, CD34, CD44, class I MHC and class II MHC was then determined. Some characteristics of 'single positive' ($CD4^+8^-$ and $CD4^-8^+$) T-lineage cells were unexpected. Amongst thymocytes, some 'immature single positives' were delineated as larger sized cells lacking cell surface CD3 and expressing low levels of class I MHC; however, in contrast with murine thymocytes these were all $CD4^+8^-$, rather than being predominantly $CD4^-8^+$. Amongst peripheral T cells, a small proportion of $CD7^-$ cells were detected, within both the $CD4^+8^-3^+$ and the $CD4^-8^+3^+$ categories. Finally, in contrast to previous conclusions, CD1a was expressed at high levels on mature ($CD4^+8^-3^+$ and $CD4^-8^+3^+$) human thymocytes, although in agreement with previous reports it was absent from peripheral T cells. CD1a is therefore a useful marker of post-selection, post-thymic T-cell maturation.

Key words: T-cell development; T-cell surface antigens; Thymocyte subpopulations

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2. Introduction

The crisis of AIDS has stimulated a closer investigation of human T cells and their development. As part of a search for potential HIV targets within the human thymus, we have analyzed thymocytes for their expression of a series of common T-cell surface markers, using three-colour flow cytometry. Most of the results obtained were in line with previously published data. However, the results on the expression of CD1 amongst thymocytes differed from the currently accepted picture [1–4]. The prevailing view that CD1 is absent from mature human thymocytes appears to be based on earlier experiments using an indirect cytotoxic approach [1], reinforced by studies on mature human peripheral T cells and by the finding that TL antigen (CD1) is not expressed by mature murine thymocytes [5]. Our direct approach indicates that CD1 is present on most mature human thymocytes, but absent from peripheral T cells. This suggests it is lost about the time of T-cell exit from the thymus, rather than at the earlier stage of positive selection.

CD1 is an antigen found predominantly on thymocytes [6] but also on Langerhans' (epidermal dendritic) cells [7] and on subsets of B cells [8]. It is normally absent from peripheral blood T cells although it has been reported to be expressed cytoplasmically after mitogenic activation [9]. CD1 has some homology with both human and mouse major histocompatibility complex (MHC) class I α and class II β chains [10], although CD1

and MHC genes are on different chromosomes [11]. CD1, like class I MHC, has binding domains for β_2-microglobulin, with which it forms non-covalently bound dimers [12]; it is also able to form intermolecular complexes with CD8 and class I MHC [13]. CD1 is in fact a group of closely related molecules (CD1a-d) encoded by separate genes [14]. These forms are antigenically distinct and can be distinguished by monoclonal antibodies (mAb) [8, 15, 16]; the mAb used in this study (OKT6) is the same as used in earlier studies on the human thymus, and recognizes CD1a [1, 15]. The biological role of CD1 is still obscure but, in line with the similarity to class I MHC, CD1b has recently been shown to present certain bacterial antigens to $CD4^-8^-$ T cells [17]. The expression of CD1 by cells of the T lineage has been shown to be linked to stages of development [1–4], a finding endorsed by our present study.

3. Materials and Methods

3.1. *Thymus tissue*

The samples of thymus tissue were provided by the Royal Children's Hospital, Parkville, Victoria, and were removed in the course of routine thoracic surgery on children with congenital heart abnormalities. The ages of the donors ranged from several days to 15 years.

3.2. *Thymocyte suspensions*

The suspension medium was a pH 7.2 HEPES-buffered balanced salt solution containing 5% fetal calf serum (BSS-FCS). A portion of thymus tissue (1.5 cm^3) was chopped with scissors then forced through a stainless-steel mesh into BSS-FCS. Dead and damaged cells were then removed by a density cut [18], selecting viable cells that floated after centrifugation at $1700 \times g$ for 10 min at 4°C in 1.086 g/cm^3 Nycodenz (Nycomed, Oslo, Norway). The viable cells were diluted, washed and resuspended in BSS-FCS.

3.3. *Peripheral blood lymphocytes (PBL)*

Donors were healthy volunteers aged 18 to 55. Blood (25 ml) was diluted 1:1 with normal saline, layered over 10 ml Ficoll-paque (Pharmacia, Piscataway, NJ, USA) and spun at $600 \times g$ for 20 min at 4°C. The upper leucocyte layer was diluted, washed and resuspended in BSS-FCS.

3.4. *Monoclonal antibodies (mAb) and fluorescent reagents*

Most hybridomas were grown and the mAb purified and conjugated in this laboratory. Phycoerythrin (PE)-conjugated anti-CD4 was purchased from DAKO, Carpinteria, CA, USA. The hybridoma clones used and their specificities are listed in Table 1. Texas red-streptavidin conjugate was obtained from Caltag Laboratories, South San Francisco, CA, USA.

3.5. *Immunofluorescent staining and flow cytometry*

In the routine procedure cells were stained simultaneously with fluorescein-conjugated anti-CD8, PE-conjugated anti-CD4, and a biotin conjugated third antibody. After washing, Texas Red-streptavidin was used as a second stage stain. Propidium iodide (PI) at 1 μg/ml was included in the final cell suspension. Six parameter flow cytometry was then carried out to determine forward and side light scatter, PI staining and the three immunofluorescent stains, using a FACStar Plus (Becton Dickinson, Mountain View, CA,

TABLE 1

Monoclonal antibodies used for thymocyte characterization.

Surface antigen	Hybridoma clone	Reference/ Source
CD1a	OKT6	1
CD2	Lym-1	18
CD3	OKT3	19
CD4	MT310; OKT4	DAKO; 19
CD7	3A1	20
CD8α	OKT8	1
CD44	Hermes-3	21
Class I MHC (HLA-A,B,C)	W6/32	22
Class II MHC (HLA-DR)	2.06	23

USA). Forward scatter and PI gates were set to exclude dead cells, debris and erythrocytes.

4. Results and Discussion

The primary division of thymocytes and PBL on the basis of CD4 and CD8 expression is shown in Fig. 1, with broken lines indicating the precise gates used to define the categories for further analysis. The expression of other markers on these gated populations is shown in Fig. 2. In PBL we examined only the two 'single positive' ($CD4^+8^-$ and $CD4^-8^+$) T cell categories. These stained brightly for CD3 (Fig. 2), as expected for mature T cells. In thymus we examined the four categories, double negative ($CD4^-8^-$), double positive ($CD4^+8^+$) and the two single positives ($CD4^+8^-$ and $CD4^-8^+$).

As shown in Fig. 2 the $CD4^-8^-$ thymocytes were mainly $CD3^-$ (immature, or non-T lineage) but with a small proportion of $CD3^{high}$ cells; some of the latter were presumably $\gamma\delta$ T cells. As expected for the immature cortical thymocyte population the $CD4^+8^+$ thymocytes expressed either very low or moderate levels of CD3. The majority of thymic single positives expressed high levels of CD3, like peripheral T cells, as expected of mature medullary thymocytes. However, a small proportion of $CD4^+8^-$ (but not of $CD4^-8^+$) thymocytes were $CD3^-$, suggesting these were immature cells. This correlated with the expression on a small proportion of $CD4^+8^-$ (but not of $CD4^-8^+$) thymocytes of low levels of class I MHC, rather than the high levels characteristic of mature T cells. These $CD4^+8^-3^-$ thymocytes were of larger size than mature thymocytes, as indicated by light scatter measurements (not shown). Immature single positives have been well characterised in the mouse thymus, as cells in transition between the $CD4^-8^-$ and $CD4^+8^+$ stages (reviewed in 25). In most mouse strains there are more $CD4^-8^+$ than $CD4^+8^-$ immature single positives, but in some strains the $CD4^+8^-$ form dominates. Our results suggest that $CD4^+8^-$ is the main immature single positive transition form in the human, in agreement with the work of others [26, 27].

The expression of T lineage surface antigens on $CD4^-8^-$ thymocytes is of interest in attempts to define a developmental sequence within this group. Most $CD4^-8^-$ thymocytes, and all other thymocytes, were shown to express CD7 (Fig. 2). It was not clear whether the $CD7^-$ $CD4^-8^-$ thymocytes were early cells, or simply non-T lineage

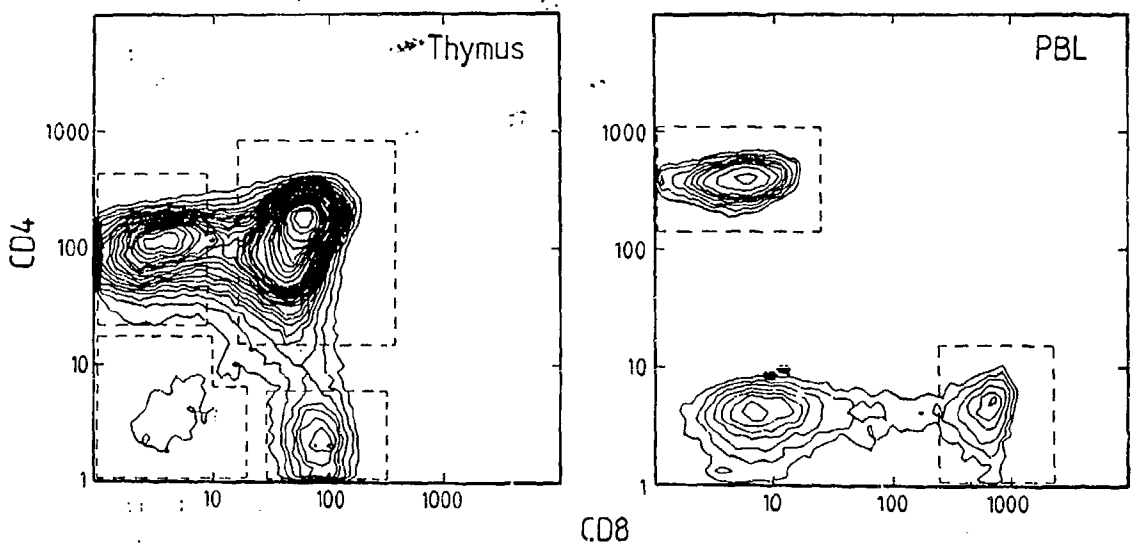

Fig. 1. The distribution of CD4 and CD8 on cells of infant human thymus and adult peripheral blood PBL. The broken lines give the gates used for subsequent analysis of subpopulations. In PBL only the $CD4^+8^-$ and $CD4^-8^+$ T-cell fractions were used for analysis. Further details are in text.

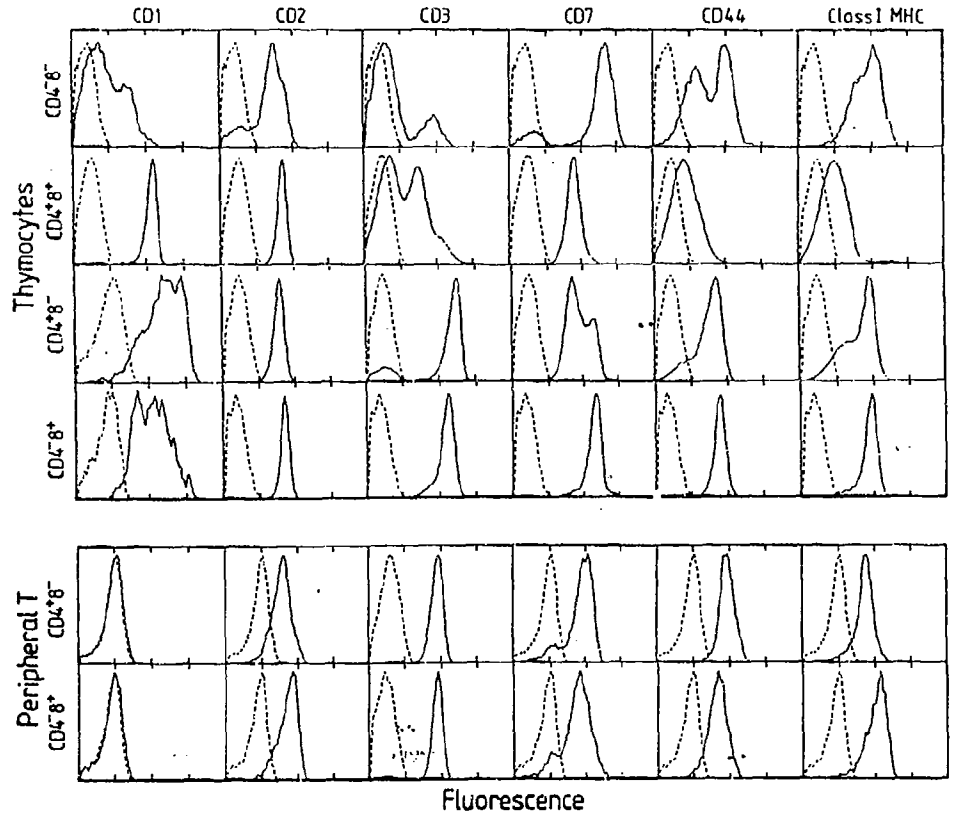

Fig. 2. The level of various surface antigens on subpopulations of human thymocytes and PBL. Results are from a single donor in each case but are representative of the results from most donors. Cells were stained in three fluorescent colours for CD4, CD8 and the antigen being studied, a separate sample being stained for each surface antigen listed. The samples were then gated for CD4 and CD8 expression, as in Fig. 1; the distribution of the third surface antigen on the gated populations is presented. The dotted lines give the background staining, representing samples stained normally for CD4 and CD8 but omitting the third antibody and using the second stage alone. The background was also gated as in Fig. 1.

cells. $CD4^-8^-$ thymocytes expressed high levels of class I MHC and some expressed very high levels of CD44, both expected characteristics which distinguish early T lineage cells from most cortical thymocytes. Most $CD4^-8^-$ thymocytes expressed CD2, but only some expressed the higher level of CD1 characteristic of cortical thymocytes. These results are in line with a sequential acquisition of markers, first CD7 then CD2 then CD1, during T-cell development. The major thymus population, the $CD4^+8^+$ cortical thymocytes were $CD1^+2^+7^+$, $CD44^{low}$, class I MHC^{low}, and expressed low and medium levels of CD3. In general these results agree with previous findings and with the developmental sequence proposed by others [1, 2, 4; reviewed in 3, 4].

Some unexpected results were seen on comparing mature T cells from PBL with the 'mature' single positive thymocytes (omitting from consideration the $CD3^-$ class I MHC^{low} 'immature' single positives discussed above). The first was the existence of a small proportion of $CD7^-$ T cells amongst PBL, but not amongst mature thymocytes; these were clearly T cells, being either $CD4^+$ or $CD8^+$, and expressing CD3 (Fig. 2). We have not examined these further, but they could be either T cells of extrathymic origin (e.g., $\gamma\delta$ T cells) or could be more mature forms such as memory T cells.

A more striking result was the presence of high

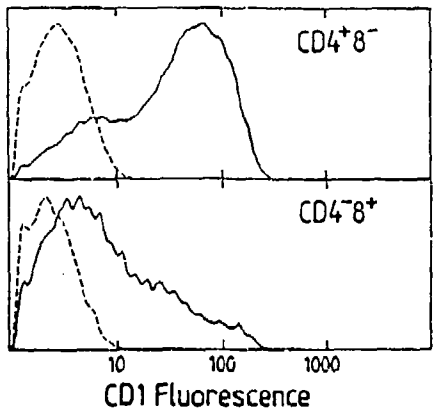

Fig. 3. The level of CD1a on mature thymocytes. The results are similar to Fig. 2, but represent an individual and less representative infant thymus sample where a proportion of the single positives were CD1a$^-$.

levels of CD1a on most CD4$^-$8$^+$3$^+$ or CD4$^+$8$^-$3$^+$ thymocytes, which contrasted with the absence of CD1a on PBL T cells (Fig. 2). This result was not in accordance with current models of T-cell development [reviewed in 3]. The level of CD1a expression varied somewhat from thymus donor to donor. Fig. 3 gives a less frequent example where a proportion of mature thymocytes were CD1a$^-$, although even in this case, as in all other samples studied, the majority were CD1a$^+$ with a spread from low to high expression. One problem with the comparison is the age discrepancy between the donors of PBL (18–55 years) and of thymus samples (1 month–15 years). However, we do not believe this is the reason for the differences between PBL T cells and mature thymocytes, since results similar to Fig. 2 were obtained comparing the thymus cells from 15 year old donors with PBL from 18 year old donors; neither set of results appeared to be age dependent. We suspect that this difference between mature thymocytes and peripheral T cells had not been noted previously simply because a direct comparison, using direct, three-colour immunofluorescent staining, had not been performed.

These results suggest that CD1 is initially maintained on human thymocytes during positive selection, despite the upregulation of the TCR-CD3 complex and the down-regulation of either CD4 or CD8. Loss of CD1 appears to be a later developmental event, occurring just before or during the time of migration of cells from the thymus to the periphery. It may serve as a useful marker of this post-selection step in the life-history of T cells.

Acknowledgements

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Assessment of CD4 Expression by Early T Precursor Cells and by Dendritic Cells in the Human Thymus[1]

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The adult mouse thymus contains a minute population of early lymphoid precursor cells that express moderate levels of CD4. We searched for a corresponding population of early T precursors in the infant human thymus, by first depleting the majority of more mature thymocytes, then using immunofluorescence and flow cytometry to analyze cells bearing a range of early T lineage markers. No discrete population of early T precursors expressing CD4 was observed, in contrast to the murine thymus. Most putative very early human thymocytes were $CD4^-8^-3^-1^-2^{lo}44^+34^+7^{hi}$ class I MHC^{hi} class II MHC^-. However, a distinct population of human thymic dendritic cells expressing high levels of CD4 was isolated. These were $CD4^{hi}8^-3^-1^-2^-44^+34^-7^-$ class I MHC^{hi} class II MHC^{hi}, and lacked markers of B cells, NK cells, or myeloid cells. They were large cells that exhibited dendritic morphology after brief periods of culture, and they were efficient stimulators of allogeneic T cells. The biologic implications of CD4 expression by thymic dendritic cells are discussed. *Journal of Immunology,* 1994, 152: 3370.

The intrathymic precursor cells that generate $CD4^+8^+$ cortical thymocytes, and thence $CD4^+8^-3^+$ and $CD4^-8^+3^+$ mature T cells, are generally considered to be within the $CD4^-8^-3^-$ ("triple negative") population, representing approximately 3% of all thymocytes (1–6). However, our laboratory has recently described an earlier and less frequent precursor population in the adult mouse thymus, and this very early precursor expresses moderate levels of CD4 (7). We have sought a similar, CD4-bearing very early precursor in the human thymus, motivated in part by the possibility that it could be a target for HIV infection.

In terms of surface markers common to mouse and human lymphoid cells, the "low CD4 precursor" population of the adult mouse thymus has the surface phenotype $CD4^{lo}$ 8^- 3^- 2^- 25^- 44^{hi}, Thy 1^{lo}, class I MHC^{hi} class II MHC^- (7). It is similar to bone marrow hemopoietic stem cells (BMSC)[3] in most but not all surface markers, and is likely to represent the immediate progeny of the cells that seed the thymus from bone marrow (7–10). It is clearly distinct from the $CD4^+8^-3^-$ "immature single positives," which are later intermediates one step removed from $CD4^+8^+$ cortical thymocytes (11–13). As with BMSC, the low CD4 precursor has TCR and Ig genes in germ-line state (7). Unlike BMSC, this thymic precursor population is unable to form erythroid or myeloid cells, but it does give rise to dendritic cells as well as T cells in the thymus, and has the capacity to form B cells in the appropriate environment (8, 9).

In searching for an equivalent cell in the human thymus, we first depleted cells bearing surface markers that should appear later in T cell development (CD3, CD8, CD1, and perhaps CD2), then analyzed for cells bearing positive markers equivalent to those on the murine early precursors (CD4, CD44, class I MHC). We also analyzed for cells bearing certain markers unique to the human system, namely CD7 (to mark T lineage cells (3–5)) and CD34 (to mark early T lineage cells and hemopoietic stem cells (5, 6)). In contrast to the adult mouse thymus we found few if any putative early T precursors expressing CD4. However, in the course of our work we isolated a human thymic dendritic cell expressing high levels of CD4.

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[3] Abbreviations used in this paper: BMSC, bone marrow hemopoietic stem cell; BSS-FCS, pH 7.2 HEPES-buffered balanced salt solution containing 2% FCS; RPMI 1640-1% FCS, RPMI 1640 medium, buffered with additional HEPES at pH 7.2 and containing 1% FCS; PI, propidium iodide; SCF, stem cell factor.

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Table I. *mAb used for thymocyte characterization*[a]

Surface Ag	Hybridoma Clone	Reference/Source
CD1a	OKT6	15
CD2	Lym-1	16
CD3	OKT3	17
CD4	MT310; OKT4	Dako; 17
CD7	3A1	18
CD8α	OKT8	15
CD14	FMC17; FMC33	19
CD16	HuNK-2	Silenus
CD19	FMC63	20
CD34	HPCA-2	Becton Dickinson
CD44	Hermes-3	21
CD56	NKH-1	Coulter
Class I MHC (HLA-A,B,C)	W6/32	22
Class II MHC (HLA-DR)	2.06	23

[a] The commercially purchased reagents were obtained from Dako (Carpinteria, CA); Silenus (Hawthorn, Victoria, Australia); Becton Dickinson (San Jose, CA); or Coulter (Hialeah, FL).

Materials and Methods

Thymus tissue

The thymus tissue was removed in the course of routine thoracic surgery on children with congenital heart abnormalities. The ages of the donors ranged from several days to 15 yr.

Thymocyte suspensions

The suspension medium was a pH 7.2 HEPES-buffered BSS containing 2% FCS (BSS-FCS). A portion of thymus tissue (1.5 cm^3) was chopped with scissors then forced through a stainless-steel mesh into BSS-FCS. Cells were washed with BSS-FCS and resuspended in RPMI 1640 medium, buffered with additional HEPES at pH 7.2, and containing 1% FCS (RPMI 1640-1% FCS). All procedures were performed at 0 to 4°C.

Isolation procedure for putative early thymocytes

The general procedure was first to deplete a thymocyte suspension of the bulk of more developed cells and non-T lineage cells, by successive cytotoxic and immunomagnetic bead procedures. This procedure was used to produce one of two populations, either CD2$^-$3$^-$8$^-$ thymocytes or CD1$^-$3$^-$8$^-$ class II MHC$^-$ thymocytes. These were then positively selected by immunofluorescent labeling and cell sorting, generally on the basis of CD4 and class I MHC expression. The detailed steps were as follows.

Cytotoxic Ab depletion

This was used only with Ab giving effective and extensive bulk depletion; anti-CD3 was avoided at this stage because of the possibility of capping rather than killing cells (14). Anti-CD2 and anti-CD8 (for CD2$^-$8$^-$3$^-$) or anti-CD8 and anti-class II MHC (for CD1$^-$3$^-$8$^-$ class II MHC$^-$) were used to coat the cells. See Table I for details of the mAb. The complement was young rabbit serum, absorbed with human thymocytes and erythrocytes, titrated, and stored frozen at −70°C. Thymocytes (4×10^8) were suspended in 30 ml RPMI 1640-1% FCS, concentrated mAb were added to titrated levels and the suspension was incubated 40 min at 0 to 4°C. Complement (9.5 ml serum) was added directly to the cell and Ab suspension, along with DNase (0.5 ml 0.1%), and mixed thoroughly. The mixture was then incubated in a 37°C water bath for 40 min after the time taken to warm up to 36°C. At intervals, samples of the cells were checked by eosin exclusion to ensure the killing was effective. The cells were then recovered by centrifugation, and dead and damaged cells removed by a density cut (24). Viable cells were selected as those that floated after centrifugation at 1700 × *g* for 10 min at 4°C in 1.086 g/cm^3 Nycodenz (Nycomed, Oslo, Norway). The viable cells were washed, suspended in BSS-FCS, and counted.

Immunomagnetic bead depletion

The mAb used for depletion were prepared as premixed mixtures of pretitrated concentration in BSS-FCS and stored at −70°C. Both mixtures contained anti-CD3, anti-CD8, anti-CD14, and anti-CD19 (see Table I). For CD2$^-$3$^-$8$^-$ the mixture also contained anti-CD2; for CD1$^-$3$^-$8$^-$ class II MHC$^-$, it also contained anti-CD1 and anti-class II MHC. Cells were spun down, resuspended in 10 μl of the mAb mixture per 10^6 cells, then incubated 40 min at 0 to 4°C. Sheep anti-mouse Ig-coated Dynabeads (Dynal, Oslo, Norway) were washed then added to the cells at an 8 beads:1 cell ratio, in a volume of 300 μl RPMI 1640-10% FCS, and the slurry incubated for 4°C on a rolling rack for 30 min. The slurry was diluted and the beads and bound cells removed with a magnet (MPC-1, Dynal). The cells were recovered by centrifugation and the bead depletion repeated with several cycles of final bead removal with the magnet. The cells were recovered and counted, and this depleted preparation used for further sorting or analysis.

Immunofluorescent staining and flow cytometry

Thymocytes were usually subjected to a two stage staining procedure. The first stage was typically fluorescein-conjugated anti-class I MHC together with phycoerythrin-conjugated anti-CD4 and a biotin-conjugated third Ab. Ab-treated cells were incubated for 20 min on ice. Cells were washed with BSS-FCS underlain with FCS. The second stage was typically Texas Red-streptavidin (Caltag Laboratories, South San Francisco, CA). Propidium iodide (PI) (1 μg/ml) was included in the final cell suspension. Six parameter flow cytometry was then carried out to determine forward and side light scatter, PI staining, and the three immunofluorescent stains using a FACStar^{Plus} (Becton Dickinson, Mountain View, CA). Forward scatter and PI staining gates were set to exclude dead cells, debris, and erythrocytes. Negative controls were included using isotype-matched irrelevant Ab.

Cytochemical staining

Staining for myeloperoxidase and dual esterase (chloroacetate esterase and α-naptholacetate esterase) was carried out by the Cell Biology Laboratory of the Royal Melbourne Hospital. Cytospun preparations of cells were stained using protocols modified from earlier work (25, 26).

Giemsa staining

Cytospun preparations were fixed in methanol-acetic acid then stained for 30 min with freshly mixed Giemsa (50 ml pH 7 citrate-phosphate buffer, 3 ml methanol, and 5 ml stock Giemsa stain).

Colony formation in agar

The cells were cultured in agar medium (AIMDM with 20% FCS and 0.3% agar) at a concentration of 500 cells/1 ml culture, in 35-mm petri dishes (27). Quadruplicate cultures were prepared with the following combinations of stimuli: 1500 U recombinant human granulocyte macrophage-CSF; 500 U recombinant human granulocyte-CSF; 100 ng recombinant human SCF; 1 U recombinant human Epo; 100 ng human SCF + 500 U granulocyte-CSF; 100 ng human SCF + 1 U Epo; 500 ng human rIL-6; 500 ng human rIL-6 + 100 ng SCF. Cultures were incubated at 37°C in a humidified atmosphere of 10% CO_2 in air and analyzed after 4, 7, and 14 days of incubation. Cultures were scored for colonies (clones >49 cells) or clusters (clones >2 cells).

Assessment of dendritic cell morphology

Cells (300) were sorted directly into 13 μl RPMI 1640-10% FCS medium in the wells of Terasaki trays, then incubated at 37°C for 2 h in an atmosphere of 10% CO_2 in air. The cells were then observed under inverted phase-contrast microscopy, 200-fold magnification, and scored for dendritic morphology (irregular shape with veils or processes).

Allogeneic MLR assays

Blood samples were obtained from healthy adult volunteers. Blood (10 ml) was diluted 1/1 with RPMI 1640 containing 10% freshly filtered human serum, layered over 10 ml Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) and spun at 500 × *g* for 20 min at room temperature. The upper leucocyte layer was diluted and washed twice before being resuspended in RPMI 1640-20% filtered human serum and 5×10^{-5} M 2-ME to produce the sample of PBL. MLC were set up in 0.2 ml medium (RPMI 1640-20% human serum) in round bottom wells of 96-well trays. The stimulator cells were either putative thymic dendritic cells (10^3–10^4/well) or PBL (10^4–10^5/well), each given 30 gray (Gy) γ irradiation. The responder cells were PBLs from a different donor, 5×10^4/well. The cultures were incubated at 37°C in a moist 5% CO_2-in-air atmosphere for 6 days. Each culture was pulsed for 8 h with 0.25 μCi [methyl-^{3}H]thymidine (67 Ci/mmol; ICN Biomedicals, Irvine, CA). The cells were then harvested onto glass microfiber sheeting, washed with water, dried, and placed in vials for liquid scintillation counting.

Results

Analysis of CD2⁻3⁻8⁻ thymocytes

Our strategy for isolating the earliest T precursors in the thymus was first to deplete most of the more developed T lineage cells, then to sort for cells bearing likely markers of early developmental stages. On the basis of our experience with murine thymocytes, we avoided depletion with anti-CD4. Based on earlier studies by others on human thymus (reviewed in Refs. 3 and 4) and on our own experience with murine thymus, we initially assumed depletion with anti-CD2 would spare the earliest precursor cells. After cytotoxic and immunomagnetic bead depletion of infant human thymocyte suspensions to eliminate cells bearing CD2, CD3, and CD8 (and also bearing many non-T lineage markers), we recovered approximately 0.2% of thymocytes, less than 0.5% of which bore normal levels of the depleted markers. These depleted thymocytes were then analyzed for expression of CD4 and of class I MHC, seeking for a $CD4^{inter}$ class I MHC^{hi} population resembling the murine "low CD4" precursor (7). Typical results are shown in Figure 1, in which the four populations used for further analysis are outlined. Many of the cells expressing high levels of class I MHC did express CD4, but at levels as high as on cortical thymocytes or $CD4^+$ T cells, rather than at the intermediate levels on the early murine T precursor. Both anti-CD4 mAb (MT310 and OKT4) gave the same bright staining.

However, these $CD4^{hi}$ class I MHC^{hi} cells seemed unlikely to be very early precursors, because they lacked CD34 expression (Fig. 2). They also did not seem to be T lineage cells, because expression of CD7 and CD1 was marginal. They expressed CD44, which is present not only on early and activated T lineage cells, but also on myeloid cells. The $CD4^-$ class I MHC^{hi} fraction seemed more likely to include the early T lineage cells, because it included cells expressing CD34, CD7, and CD44, but lacking CD1 (Fig. 2). The cells expressing the lower levels of class I MHC seemed likely to be more developed T lineage cells. In particular the $CD4^{hi}$ class I MHC^{lo} population, which expressed CD1 and CD7 but little CD34 or

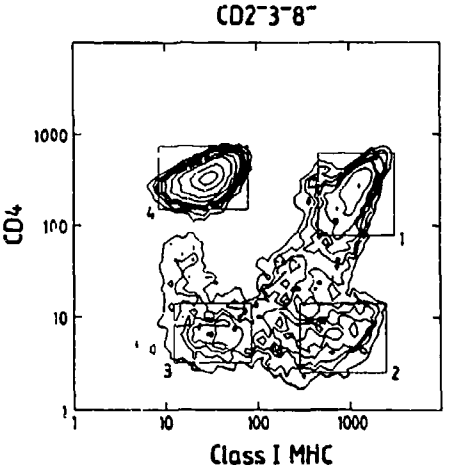

FIGURE 1. The distribution of CD4 and class I MHC on human thymocytes depleted of cells bearing CD2, CD3, and CD8. Further details of the preparation are in *Materials and Methods*. The regions used for subsequent subpopulation analysis are outlined by fine lines. These results on an individual thymus sample were typical of 12 such experiments.

CD44, had many of the properties of more mature thymocytes, despite the apparent absence of CD2 or CD3. The recovery of this population was highly variable, so we suspect it represented the tiny fraction of late thymocytes that escaped the depletion procedures.

Nature of the $CD4^{hi}$ class I MHC^{hi} population

It seemed the $CD4^{hi}$ class I MHC^{hi} population (other markers $CD1^-2^-3^-8^-34^-44^+$) might not be T lineage cells, despite our attempts to deplete other minor thymic lineages. In particular, its very high forward and side light scatter characteristics indicated it did not consist of lymphoid cells. Accordingly we analyzed the population for markers of monocytes or macrophages (CD14), NK cells (CD16, CD56), and B cells (CD19). In these studies the lineage-specific mAb were omitted from the depletion procedure, in case these markers were merely blocked or capped off. However, the cells were clearly negative for all of these markers (Fig. 3). Further evidence that these were not myeloid cells came from histologic staining of the sorted cells. All cells were negative for α-naptholacetate esterase and for myeloperoxidase, and only 1 to 2% were positive for chloroacetate esterase. Finally, to determine if these cells were immature precursors of the myeloid or erythroid lineages, they were cultured in agar with various mixtures of cytokines, under conditions allowing growth and differentiation of precursor cells. However, no colonies or even clusters of cells grew in the agar plates.

The one positive surface marker giving a clue to the nature of these $CD4^{hi}$ cells was their expression of very high levels of class II MHC. This, together with their expression of high levels of class I MHC and their large size,

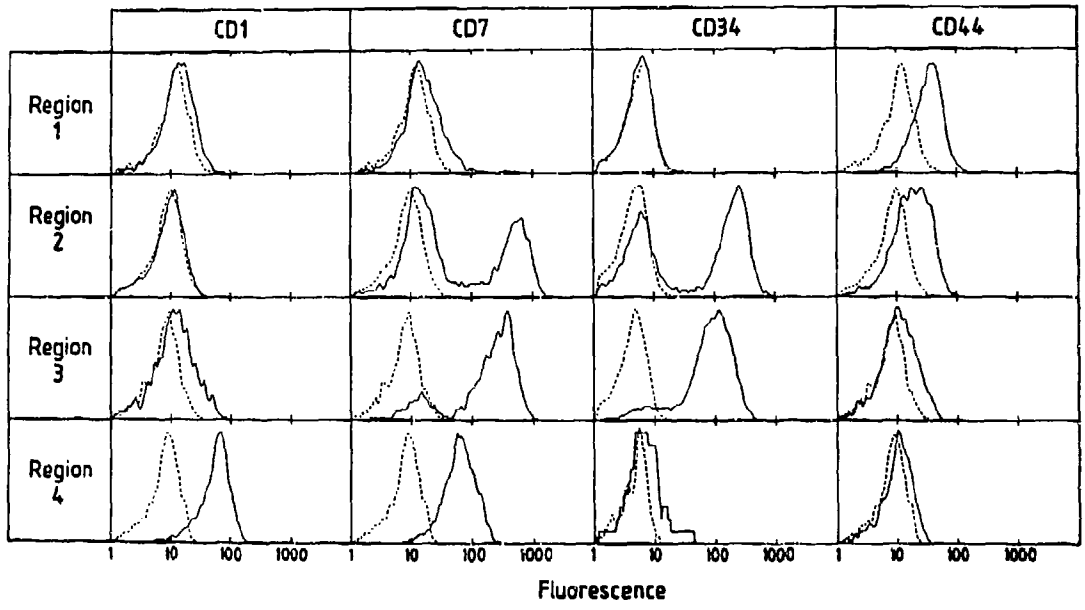

FIGURE 2. The levels of various T cell differentiation Ag on subpopulations of human $CD2^-3^-8^-$ thymocytes. The four regions analyzed correspond to those outlined in Figure 1. The $CD2^-3^-8^-$ thymocytes were stained in three fluorescent colors for CD4, class I MHC, and the Ag heading each column, a separate sample being stained for each marker listed. The samples were then gated for CD4 and class I MHC expression, as in Figure 1. The broken lines give the background staining, representing samples stained for CD4 and class I MHC and second stage reagents, omitting only the third Ab; background distributions were also gated as in Figure 1. Results are for an individual thymus sample but were typical of three such analyses.

suggested they were dendritic cells. This was not immediately apparent from Giemsa-stained cytospin preparations, in which most cells appeared round, although some showed cytoplasmic extensions (Fig. 4). In the main the cells were large, usually with extensive pale staining cytoplasm, often with vacuoles but with no signs of phagocytic activity. Nuclear shape varied from round to eliptical. Because these cells had been subjected to a long isolation procedure, much of it in the cold, they were incubated for several hours at 37°C in culture medium to recover normal morphology, then examined by phase-contrast microscopy (Fig. 5). A high proportion (50–70%) then assumed an irregular shape, with cytoplasmic processes and "veils" typical of dendritic cells.

Dendritic cells are extremely efficient stimulators of allogeneic T cells in an MLC (28). Accordingly, these $CD4^{hi}$ MHC^{hi} thymic cells were compared with PBLs in their ability to stimulate allogeneic PBLs. The extent of responder lymphocyte proliferation was similar to that seen with PBL stimulators, but 10- to 100-fold fewer cells were required for peak stimulation (Fig. 6). This functional assay suggested they were indeed dendritic cells.

Surface phenotype of putative early T precursors

Inasmuch as the $CD2^-3^-8^-$ fraction of infant human thymus included no obvious subgroup of CD4-bearing early T precursors, and because some recent studies have suggested even the earliest human thymocytes express CD2 (5), we considered the possibility that we had depleted the earlier precursors from our preparations. Accordingly, we used anti-CD1 instead of anti-CD2 to remove the more developed T lineage cells, and used additional depletion with anti-class II MHC to remove the CD4-bearing dendritic cells. The analysis of these $CD1^-3^-8^-$ class II MHC^- thymocytes (approximately 0.5% of original thymocytes) is shown in Figure 7. The class I MHC^{hi} fraction now included no cells expressing CD4. The class I MHC^{lo} cells included cells expressing CD4, but as before the recovery of these was variable. Because these cells expressed CD7, but no CD34 or class II MHC, it seemed likely that, as before, they were more mature T lineage cells that had evaded the depletion procedure.

It seemed from this analysis that the putative earliest T lineage cells in the thymus did not express CD4. To assess this more thoroughly, the $CD1^-3^-8^-$ class II MHC^- fraction was gated on the basis of anti-CD34 fluorescent staining, using CD34 expression as a criterion to define the earliest precursor cells (5, 6, 13). As shown in Figure 8, the $CD34^+$ cells all expressed high levels of CD7, suggesting that they were all T lineage cells and that we had now succeeded in depleting all other lineages from the preparation. These putative early T lineage cells also expressed CD2, although at lower levels than most thymocytes. However, they were all clearly $CD4^-$, in contrast to cells in the $CD34^-$ fraction.

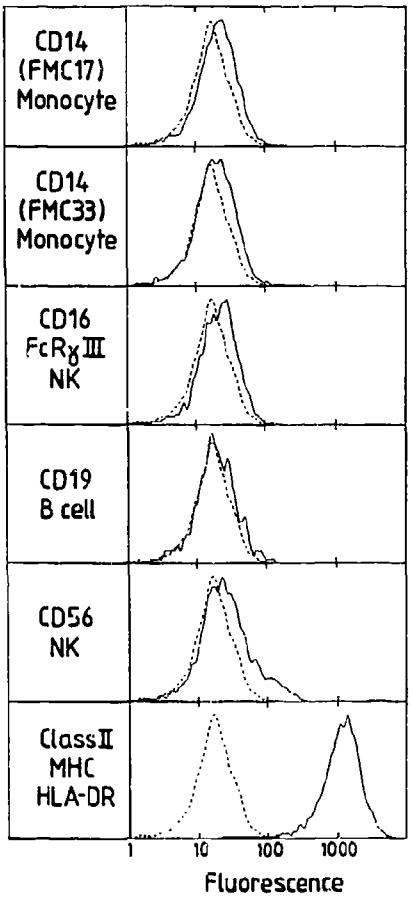

FIGURE 3. Expression of various non-T lineage markers on subpopulations of $CD2^-3^-8^-$, $CD4^{hi}$ class I MHC^{hi} thymocytes. The cells analyzed correspond to region 1 of Figure 1. In the preparation of $CD2^-3^-8^-$ thymocytes for these analyses, depletion of cells bearing CD14 and CD19 was omitted. The histograms are derived from three color staining for CD4, for class I MHC and for the marker of interest, with gating for the $CD4^{hi}$ class I MHC^{hi} group, as in Figure 2. The broken line gives the background fluorescence for the gated subpopulation, as in Figure 2. Results are from an individual thymus sample but were typical of two to three such analyses.

FIGURE 4. The morphological appearance of the freshly separated $CD2^-3^-8^-$, $CD4^{hi}$ class I MHC^{hi} cells. Cells corresponding to region 1 of Figure 1 were sorted, cytospin preparations made and stained with Giemsa. Magnification is 1700-fold. Most cells were large but showed a range of morphologic appearances, from round cells with round nuclei, to cells with eliptical nuclei and cells with cytoplasmic extensions. Three experiments gave similar results.

We also checked to see if low levels of CD8, rather than CD4, might be present on the earliest T precursors in the human thymus. In these experiments the depletion procedures were similar, but anti-CD4 was substituted for anti-CD8. However, analysis of these $CD1^-3^-4^-$ class II MHC^- preparations gave no evidence for a CD8-bearing class I MHC^{hi} $CD7^+$ $CD34^+$ population (data not shown). Overall the putative earliest T precursor populations in the infant human thymus appeared to be $CD4^-8^-$.

Discussion

The earliest T precursor cells in the thymus would be expected to represent less than 0.1% of all thymocytes (7), a level similar to that of many minor non-T cell lineages, including dendritic cells. Our study serves to emphasize the need to control for non-T lineage cells when isolating early thymocytes, because our initial search for the earliest T precursors led to the isolation of a dendritic cell. This was in part due to the use of anti-CD2 in the initial depletion procedure, which enriched for dendritic cells but excluded many early precursors. Most human thymic early T precursor cells appear to express CD2 (5), in contrast to mouse thymic early T precursors (29). Human thymic dendritic cells do not express CD2 (30), again in contrast with mouse thymic dendritic cells (31). Our results indicate that for the human thymus, the more developed T lineage thymocytes should be depleted using Ab against the later surface markers CD1, CD3, CD4, and CD8, but not against CD2. To deplete non-T lineage cells not only should Ab against B cells, macrophages, granulocytes, NK cells, and erythrocytes be included, but also Ab against class II MHC, to eliminate dendritic cells.

Our primary objective in this study was to determine whether the earliest T lineage cells in the human thymus express CD4, as we have found for the adult mouse thymus (7). Lacking a suitable functional assay we have identified putative early T precursors both by analogy with murine thymic T precursors, and on the basis of markers expressed by human BMSCs. We reasoned that human

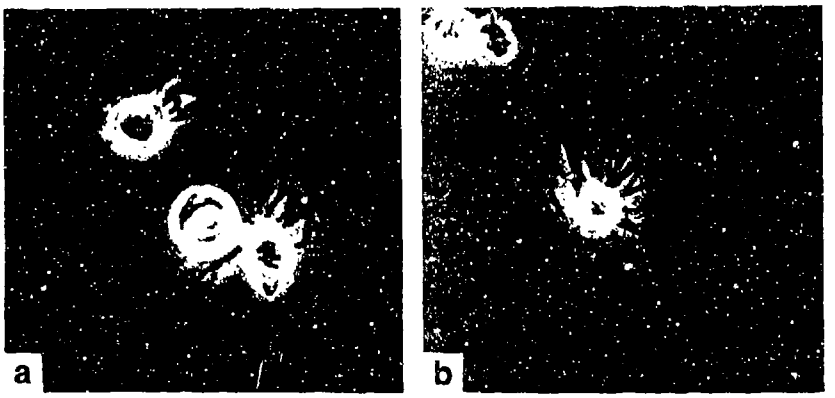

FIGURE 5. The morphologic appearance of the CD2 3 8 CD4^{hi} class I MHC^{hi} cells, after culture for 3 to 5 h. Cells equivalent to region 1 of Figure 1 were sorted and incubated at 37°C in simple culture medium. They were then examined under phase-contrast microscopy. *a*, A typical example after 3 h incubation. Magnification is 1000-fold. *b*, A more florid example apparent at 5 h, along with increasing cell death. Magnification is 700-fold. Five experiments gave similar results.

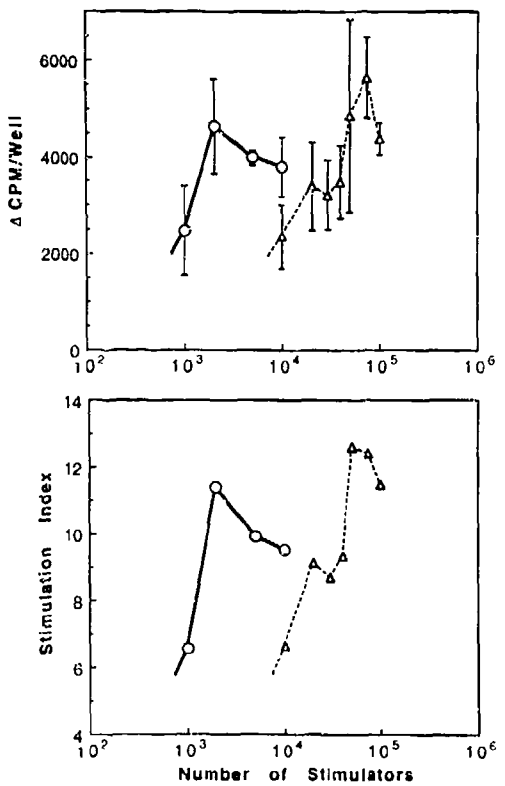

FIGURE 6. Stimulation of allogeneic PBL with the putative dendritic cells isolated as region 1 of Figure 1. The stimulation achieved is compared to that given by PBL (broken line). Results are presented as absolute counts above the background (Δ cpm/well) and as a ratio of stimulated to unstimulated cultures (stimulation index). A second experiment gave similar results.

thymic early T precursor cells are likely to express CD34, CD7, CD44, and high levels of class I MHC, but should not express CD1 or surface CD3 (3–6). On the basis of this identification, we find most of these putative early thymocytes do express CD2, in agreement with Terstappen et al. (5), although at lower levels than on the majority of thymocytes. However, we have not found a significant subpopulation of the CD34$^+$7$^+$1 3 class II MHC thymocytes that expresses either CD4 or CD8, so the earliest thymic precursors appear to be CD4 8 as most current models suggest (3–6). We doubt if this difference from the mouse thymus is simply due to the age of the thymus donors, since the "low CD4 precursor" population is discernible in the young adult mouse and our human thymus samples came from infants up to 15 yr old.

The absence of detectable CD4 on these early human thymocytes seems to be in some conflict with the results of Schnittman et al. (32), who found a low level of CD4 expression even on "CD4 8 3 " human thymocytes, a level sufficient for infection with HIV virus. Their preparations may have included some non-T lineage cells. However, the results are not necessarily in conflict because CD4 8 3 thymocytes later than those we were selecting, namely those that are CD34 and CD1$^+$ and close to the CD4$^+$8$^+$ state, may well express low levels of CD4. Human thymocytes appear to express CD4 before CD8 during development, resulting in a discrete CD4$^+$8 "immature single positive" subpopulation as a precursor of CD4$^+$8$^+$ thymocytes (12, 13). However, this relatively late CD4-expressing human T precursor cell is quite distinct from the very early CD4^{lo} precursor cell found in the adult mouse thymus.

The surprising result of our study is the identification of a population of CD4^{hi} cells as thymic dendritic cells. These cells displayed dendritic morphology after brief periods of culture. They were effective stimulators of allogeneic T cells, a hallmark of dendritic cells. These large cells lacked the characteristic markers of T or B lymphocytes, NK cells, monocytes, or granulocytes. Their surface phenotype was CD4^{hi}8 3 1 2 7 34 44$^+$ class I MHC^{hi} class II MHC^{hi}. Certain thymic medullary dendritic cells have been reported to be CD1$^+$, as are skin Langerhans' cells (3), although others have reported CD1 thymic DC (30). The CD4^{hi} thymic dendritic cell population we have isolated is CD1 . Our separation procedure, not originally designed for dendritic cell isolation,

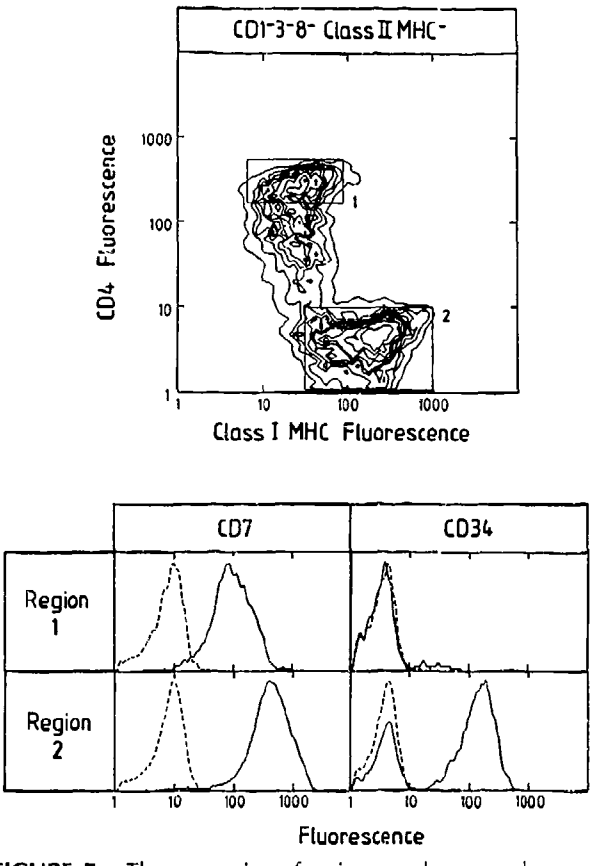

FIGURE 7. The expression of various markers on subpopulations of CD1⁻3⁻8⁻ class II MHC⁻ thymocytes. Thymocytes were depleted of cells bearing these markers (and various non-T lineage markers) as described in *Materials and Methods*. They were then stained in three fluorescent colors for CD4, class I MHC, and either CD7 or CD34. The distribution of CD4 and class I MHC is shown in the upper diagram for direct comparison with Figure 1. The two regions gated for further analysis of CD7 and CD34 expression are outlined in fine lines. Note the absence of $CD4^{hi}$ class I MHC^{hi} cells. Results are from an individual thymus sample, typical of three such analyses.

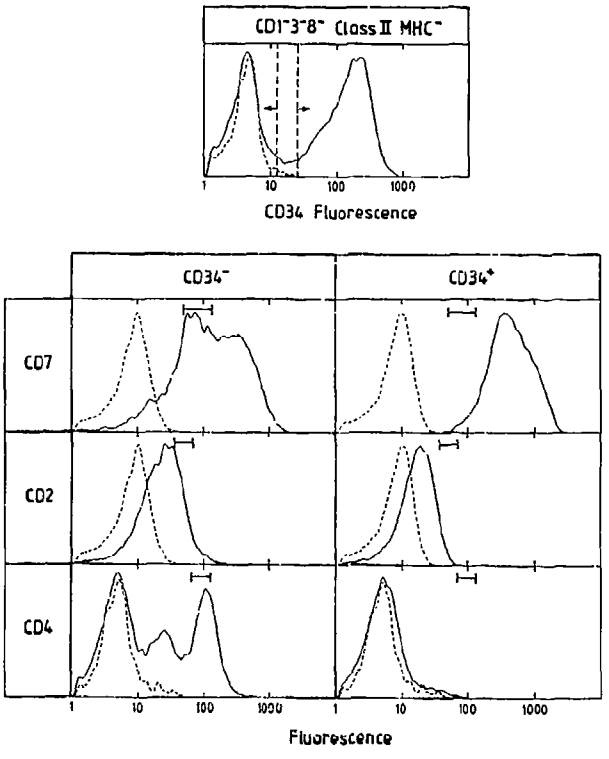

FIGURE 8. The level of CD4, CD2, and CD7 on CD1⁻3⁻8⁻ class II MHC⁻ thymocytes further gated for CD34 expression. The thymocytes were depleted as in Figure 7, then stained in two fluorescent colors for CD34 and either CD7, CD2, or CD4. The gates used to separate $CD34^+$ and $CD34^-$ cells are shown in the upper diagram. Background fluorescence (CD34 stain and second stage reagent alone) is given as a broken line. For comparative purposes the level of peak staining of unfractionated thymocytes is indicated (|——|). This single experiment confirmed the results of other multiparameter staining and cross-correlation experiments.

did not produce any $CD1^+$ class II MHC^+ $CD7^-$ dendritic cells.

At this stage we are not sure if the expression of high levels of CD4 is a characteristic of all human thymic dendritic cells, or only of a special subgroup; we did note some cells with lower levels of CD4 (Fig. 1). Most previous reports of human dendritic cells have indicated they are $CD4^-$ (33), or give only marginal staining with anti-CD4 (34, 35). However, a recent study indicates that freshly isolated human blood dendritic cells express CD4, which is lost on further maturation (36). Some Langerhans' cells (epidermal dendritic cells) are $CD4^+$, and their expression of CD4 can be induced by IFN-γ (37). In one research report human thymic dendritic cells were claimed to be $CD4^-$ (30), although a recent review (38) indicates they are $CD4^+$. Mouse thymic dendritic cells show low staining with anti-CD4 (31). The striking feature of our results is that the staining of human thymic dendritic cells with anti-CD4 is as strong as for thymocytes or T cells.

This expression of T cell markers on thymic dendritic cells may be more than coincidental. We have recently found that the earliest precursor population in the adult murine thymus, which originally appeared to be lymphoid restricted (8), gives rise to both T lineage and dendritic cell progeny when transferred to an irradiated recipient thymus (9). Within the thymus, dendritic cells and T lineage cells have a similar lifespan and develop in parallel from what may be a common precursor cell (9). Either the common inductive environment, or the common precursor origin, could be the basis of the shared surface Ag. The biologic function of these characteristic T cell molecules on the surface of dendritic cells is at present obscure. However, the high levels of CD4 on the human thymic dendritic cell

does suggest it could be a target for the HIV virus. Experiments to test this possibility are in progress.

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CD4 and CD8 expression by human and mouse thymic dendritic cells

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CD4 and CD8 expression by human and mouse thymic dendritic cells

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1. Summary

Dendritic cells (DC) from human and mouse thymus were compared. DC from both sources were isolated by digestion with collagenase, disruption of cellular complexes with a chelating agent, selection of light density cells, immunomagnetic bead depletion of other cell types (without depletion with anti-CD4 or anti-CD8) and finally sorting for cells expressing high levels of class II MHC. Yields of DC from human and mouse thymus were comparable (around 1 DC/10^3 thymocytes), they displayed similar DC morphology, and both showed strong expression of CD11c. DC from the human thymus all expressed very high levels of CD4 but low levels of CD8. In contrast, DC from the mouse thymus expressed high levels of CD8 but only low levels of CD4. Human thymic DC were also substantially larger than mouse thymic DC. The biological significance of CD4 and CD8 expression by DC is discussed in view of this major species difference and the possibility that human thymic DC may be targets for HIV infection.

2. Introduction

The dendritic cells (DC) found in lymphoid tissues are specialized for efficient interaction with T lymphocytes and for presentation to them of peptide antigens [1,2]. The array of molecules on the surface of DC reflects this functional specialization and includes high levels of class I and class II MHC, many cell adhesion molecules and molecules which serve as co-stimulators of T cells [1–4]. DC found within the thymus are generally believed to effect negative selection, the elimination of developing T cells potentially responsive to self-peptides presented on self-MHC [5,6]. Most DC within the thymus are in a close physical association with developing T cells and, on release from the tissue, this association takes the form of thymic 'rosettes', a central DC with 10–15 attached thymocytes [7,8]. Recent studies from this laboratory [9] have revealed a close developmental as well as physical relationship between murine thymic DC and T-lineage thymocytes, with a possible common intrathymic precursor. This developmental relationship may help explain the high incidence of characteristic T-cell surface markers on mouse thymic DC [10].

One T-cell marker expressed at high levels on a subgroup of mouse DC is CD8 [10–14]. We have demonstrated that this is formed by the DC themselves and consists mainly of the $\alpha\alpha$ homodimer rather than the $\alpha\beta$ heterodimer characteristic of most T cells [10]. Most mouse thymic DC express CD8 [10]. In contrast, we have recently isolated a population of DC from the human thymus which expresses high levels of CD4 [15]. This is in conflict with an earlier report suggesting human thymic DC are $CD4^-$ [16].

It seemed that such differences might simply result from different isolation procedures selecting different subpopulations of DC, as we have demonstrated previously [10]. Even in our own studies suggesting a species difference, very different procedures had been used for isolating mouse [10] or human [15] thymic DC. Accordingly we have made a side-by-side

Key words: Dendritic cell; Human dendritic cell; Murine dendritic cell; Thymic dendritic cell; CD4; CD8

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comparison of mouse and human thymic DC, using basically the same isolation procedure. The procedure used was designed to extract a high proportion of all thymic DC, to release DC from rosette complexes, and to avoid any preselection for CD4 or CD8 expression. The results indicate that there is indeed a fundamental species difference. Most mouse thymic DC express high levels of CD8 but low levels of CD4 whereas most human thymic DC express high levels of CD4 but low levels of CD8.

3. Materials and Methods

3.1. Mouse thymus tissue

Thymuses were removed from inbred specific-pathogen-free C57BL/6J Wehi mice bred at The Walter and Eliza Hall Institute animal facility. Most samples were from 6-week-old mice but 4-week-old mice were also used where indicated.

3.2. Human thymus tissue

Thymus tissue was removed during routine thoracic surgery on children with congenital heart abnormalities. Most samples came from children aged 4 days to 2 years, but tissue from a child aged 10 years was also studied.

3.3. Suspension media

Media for use with murine cells were at 'mouse osmolarity', 308 m.osmol or equivalent to 0.168 M NaCl. Media for use with human cells were at 'human osmolarity', 269 m.osmol or equivalent to 0.147 M NaCl. For use with human cells fetal calf serum (FCS) was first diluted with 15 ml of H_2O/100 ml of FCS to produce 'human osmolarity'. For the initial tissue dispersal and digestion the medium was RPMI-1640 with additional pH 7.2 HEPES buffer, and containing 2% FCS (RPMI-1640 FCS). To avoid any reassociation of DC with T cells in the later stages of separation the basic medium lacked divalent metals and consisted of NaCl and KCl (molar ratio, 40:1), with pH 7.2 HEPES buffer and including 5 mM EDTA (EDTA BSS). To this was normally added 5% FCS which had divalent metals chelated by the addition of 1 ml of 0.1 M EDTA (pH 7.2)/10 ml of serum (EDTA BSS FCS). The media for density separation of DC were prepared by diluting stock solutions made from solid Metrizamide or Nycodenz (both from Nycomed Pharma, Oslo, Norway) in water (0.308 M for mouse cells; 0.294 M for human cells), with EDTA–BSS (of mouse or human osmolarity). The final density of the separation media at 4°C was 1.071 g/cm^3 Metrizamide for mouse DC, 1.065 g/cm^3 Nycodenz for human DC, verified by weighing in a calibrated weighing bottle. The actual measured osmolarity obtained by this oversimplified approach was 276 m.osmol for 'mouse osmolarity' Metrizamide, 256 m.osmol for 'human osmolarity' Nycodenz; density separation was carried out using these slightly hypotonic media, rather than attempting to adjust the media to the 'correct' osmolarity. After separation and staining of the cells, the final suspension medium was a full balanced salt solution (Na/K/Ca/Mg in the molar ratio 121:3:2:1) buffered to pH 7.2 with HEPES and containing 5% FCS (BSS FCS).

3.4. Isolation of mouse thymic DC

The procedure was similar to that published previously [10]. All media used were iso-osmotic with mouse serum. About 15 thymuses, free of extraneous tissue, were cut into very small fragments with sharp scissors. Instead of washing out free lymphoid cells prior to digestion as in our normal procedure [10], the fragments were directly suspended in 10 ml of RPMI-1640 FCS containing collagenase (1 mg/ml; type II, Worthington Biochemicals, Freehold, NJ) and DNAse (0.02 mg/ml; grade II bovine pancreatic DNAse I; Boehringer-Mannheim, Mannheim, Germany). The fragments were digested with continuous agitation for 20–30 min at room temperature (22°C); pipetting in and out of a wide-bore Pasteur pipette was used to help release cells from the fragments. Most fragments broke down but, although the DC were released, many small stromal fragments, including most epithelial cells, remained undigested. To dissociate thymic rosettes, EDTA was added (1.0 ml of 0.1 M EDTA, pH 7.2) and incubation with agitation continued for 5 min. Undigested stromal fragments were then removed by passing through a fine sieve. All remaining procedures were at 0–4°C. The cells were recovered from the digest by centrifugation at 500 *g* for 7 min. The cell pellet was immediately dispersed in 1.071 g/cm^3 Metrizamide medium and a low-density fraction selected after centrifugation for 10 min at 1700 *g*, according to the density cut procedure described in detail elsewhere [17]. The low-density cells (3–4.5% of the original thymocytes) were diluted in EDTA BSS FCS and recovered by centrifugation. The cells were then incubated for 30 min at 0–4°C with a cocktail of mAb consisting of: anti-

CD3, KT3-1.1; anti-Thy-1.2, 30-H12; anti-IL-2Ra, PC61; anti-Gr-1, RB68C5; anti-Mac-1a, M1/70.15; anti-macrophage, F4/80; anti-B220, RA36B2; anti-erythrocyte, TER119; anti-FcRII, 2.4G2. Full details of the mAb are given elsewhere [10]. Note that this cocktail did not include anti-CD4, usually included to prepare DC [10], nor did it include anti-CD8. The mAb were all pre-titrated and most were used at minimal saturating levels (as for immunofluorescent staining); however, anti-Thy-1, anti-Mac-1a and anti-FcRII, which are expressed at low levels on DC, were used at a subsaturating level (40% of the normal staining level) to prevent depletion of DC bearing low levels of these antigens. After incubation the cells were washed by layering over and spinning through FCS–EDTA. The cells were then depleted twice with anti-Ig-coated magnetic beads, using a 1:1 mixture of anti-rat Ig and anti-mouse Ig beads (Dynabeads, Dynal, Oslo), first at a 7:1 then at a 3:1 bead-to-cell ratio. The bead and cell mix was in the form of a concentrated slurry in EDTA–BSS–FCS, and was incubated at 4°C for 20 min with continuous slow rotation in a small sealed tube. The mix was then diluted with 5 ml of EDTA–BSS–FCS and the beads with attached cells removed magnetically, using 3 removal cycles after the final bead treatment. This final preparation contained 29–55% DC as defined by high levels of class II MHC, together with some immature DC expressing lower levels of class II MHC. Pure DC were then derived from this by cell sorting, or were analyzed by selective gating, for cells with high forward light scatter ($> 1.4\times$ the peak value for mouse thymocytes), and high side light scatter, and expression of high levels of class II MHC, as detailed previously [9,10].

3.5. Isolation of human thymic DC

The procedure followed closely that for mouse thymic DC, but using media iso-osmotic with human rather than mouse serum. The thymus tissue was carefully dissected free of the fibrous capsule and then cut into small fragments with sharp scissors. The fragments were digested with collagenase-DNAse in RPMI-1640–FCS medium as for the mouse thymus preparation, but using 2 mg/ml of collagenase. Complexes of DC with T cells were broken up by EDTA treatment, as for the mouse thymic DC preparation. Light density cells were selected with a Nycodenz density cut, using medium of density 1.065 g/cm^3 at 4°C; 6–8% of the original thymocytes were in this fraction. Immunomagnetic bead depletion of non-DC was then carried out as for mouse thymic DC, but using the following mAb: anti-CD3, OKT3; anti-CD7, 3A1; anti-CD2, LYM-1; anti-CD19, FMC63; anti-CD14, FMC33. Full details of the mAb are given elsewhere [15]. Note that neither anti-CD4 nor anti-CD8 was used for depletion in these experiments, although anti-CD8 (OKT8) would usually be included to improve DC purity. Since all mAb were of mouse origin, only anti-mouse Ig-coated Dynabeads were used to remove the coated cells, following the procedure for mouse thymic DC. The final preparation contained 24–72% DC with high levels of class II MHC, together with some immature DC expressing lower levels. Pure DC were then either isolated by cell sorting, or were analyzed by selective gating, for cells with high forward light scatter ($> 1.8\times$ the peak value for human thymocytes), and high side light scatter, and expressing high levels of class II MHC as detailed previously for mouse DC [9,10].

3.6. Immunofluorescent staining

Full details of immunofluorescent staining procedures and the mAb used have been given elsewhere [10,15]. Human DC were usually pre-incubated with mouse immunoglobulin to reduce non-specific staining; this was not necessary for the mouse DC preparations. Human DC were stained with fluorescein isothiocyanate (FITC)-conjugated anti-class II MHC (2.06) together with either phycoerythrin (PE)-conjugated anti-CD11c (Leu-M5), or PE-conjugated anti-CD4 (MT310), or biotin-conjugated anti-CD4 (OKT4), or biotin-conjugated anti-CD8 (OKT8); the biotin conjugates were then followed by a second stage PE–avidin stain. Mouse DC were stained with either FITC-conjugated or an allophycocyanin (APC)-conjugated anti-class II MHC (M5/114 for C57BL/6 mice), together with either PE-conjugated anti-CD4 (GK1.5), or PE-conjugated anti-CD8a (53-6.7), or FITC-conjugated anti-CD11c (N418). The other mAb used were as described previously [10,15]. Propidium iodide (PI), 1 mg/ml, was included in the final cell wash to label any dead cells.

3.7. Flow cytometry and cell sorting

Flow cytometric analysis was carried out either on a FACScan (Becton Dickinson, San Jose, CA) using channels FL-1 for FITC, FL-2 for PE and FL-3 for exclusion of PI-positive cells, or on a FACStar Plus (Becton Dickinson, San Jose, CA) using FL-1 for FITC, FL-2 for PE, FL-4 for APC, and FL-3 for exclusion of PI-positive cells. Cell sorting was car-

ried out using the FACStar Plus instrument.

3.8. *Phase-contrast microscopy*

Putative DC were sorted on the basis of high class II MHC expression and light-scatter characteristics, and then incubated at 2.5×10^6 cells/ml in RPMI-1640–FCS (10%) medium in a siliconized glass tube for 1–3 h at 37°C, in an atmosphere of 10% CO_2 in air, to allow the DC to recover their typical morphology [10,15]. The cells were then mixed and the suspension placed in prewarmed slide chambers. The chambers were prepared by fastening square coverslips to microscope slides by double-sided adhesive tape on two opposite edges; after filling the chambers the remaining edges were sealed with nail polish. The chambers were kept warm in the incubator until examination under phase-contrast microscopy using a 40× objective.

4. Results and Discussion

In order to allow a direct comparison of thymic DC from human and mouse, the DC enrichment procedure was designed to isolate as many DC as possible from thymic tissue without preselection of particular DC subpopulations. Thus we used a mild collagenase digestion to release bound DC from stromal fragments, but we also included the free DC in the preparation. We also used EDTA treatment to release DC from complexes with thymocytes. Our procedure avoided any adherence step involving incubation at 37°C, since this can bias the preparation to particular DC subpopulations and lead to changes in surface phenotype [10]. Normally during the immunomagnetic bead depletion step we would treat murine DC preparations with anti-CD4 and human DC preparations with anti-CD8, to ensure maximum DC purity and minimum T-cell contamination. However, in the present study we avoided any depletion with anti-CD4 or anti-CD8, to ensure the comparison of human and murine DC was unbiased in this respect. Using this modified procedure as described in Materials and Methods, the average yield of class II MHC^{hi} DC from human thymus was 2.1 (0.5–4.0) per 10^3 original thymocytes. This yield was about 3-fold higher than in our earlier study of human thymic DC [15] and the procedure was 2–3 h shorter, so it represented a significant improvement. From the mouse thymus the average yield of class II MHC^{hi} DC was 0.7 (0.2–2.8) per 10^3 original thymocytes.

After sorting or gating for large-sized cells expressing high levels of class II MHC, the cells purified from both mouse and human thymus had the surface antigenic phenotype expected for thymic DC. As shown in Fig. 1, the class II MHC^{hi} cells from the mouse thymus were more than 96% positive for the DC marker CD11c (N418) [18], which was expressed at high levels. The class II^{hi} cells from the human thymus were more than 85% positive for the equivalent CD11c marker; however, the level of expression ranged from moderate to high, so did not provide as clear a distinction from other cell types as was obtained with the mouse DC. Nevertheless, with both sources 2-color sorting for cells bearing high levels of both class II MHC and CD11c was an effective means of ensuring high DC purity, although probably with selection for the most mature DC. The putative DC from the mouse thymus were also class I MHC^{hi}, $CD44^{hi}$, and expressed moderate levels of Thy-1 (data not shown), as described previously [10]. The putative DC from human thymus were also class I MHC^{hi}, $CD44^{+}$, $B7/BB\text{-}1^{+}$,

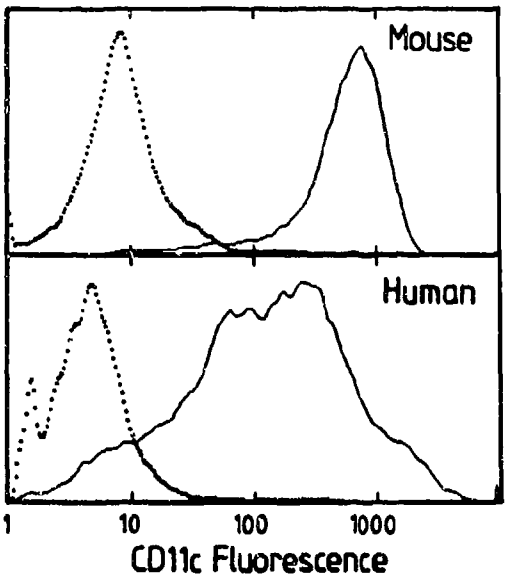

Fig. 1. The expression of CD11c by mouse thymus and human thymus DC. The DC were isolated as described in Materials and Methods then stained in 2 fluorescent colors for class II MHC (blue APC fluorescence for mouse or green FITC fluorescence for human) and for CD11c (green FITC fluorescence for mouse and red PE fluorescence for human). The samples were gated for PI fluorescence to exclude dead or damaged cells, and gated to include cells with very high class II MHC fluorescence together with high forward and side scatter. The fluorescence distribution of CD11c on these gated cells is shown as a solid line. The dotted line is the background fluorescence omitting only the CD11c reagent. Results are typical of 20 such experiments for mouse DC, 4 experiments with human DC.

CD1$^-$2$^-$3$^-$7/14$^-$16$^-$19$^-$34$^-$56$^-$ (data not shown) exactly as we had found using our previous isolation procedure [15]. In this previous study such cells were shown to be efficient stimulators of allogeneic peripheral blood T cells [15].

The isolated cells did not immediately exhibit a DC appearance, since most had rounded up during the long isolation at 4°C. After 1–5 h of culture in simple media to recover normal surface configuration, about 85% of the high class II MHC cells isolated from the human thymus, and about 40% of the corresponding cells isolated from the mouse thymus, exhibited clear DC morphology, with cytoplasmic extensions and a convoluted shape (Fig. 2). Many of the cells which remained round may have exhibited DC morphology with longer culture periods, but after 4 h many of the cells began to disintegrate. The cells from the human thymus always exhibited a more florid DC morphology with many more cytoplasmic extensions. They also appeared larger than the mouse thymic DC (Fig. 2). This was confirmed by light scatter measurements. Using as a control a thymocyte suspension of the same species in the appropriate iso-osmotic medium, set at 50 forward light scatter units, the modal value of forward light scatter was 80 for mouse thymic DC, 105 for human thymic DC. Thus human thymic DC were larger both in absolute terms and in relation to their own thymocytes.

The DC from mouse and human thymus differed markedly in their pattern of CD4 and CD8 expression, despite the use of similar isolation procedures which lacked any obvious subpopulation selection bias. Most mouse thymic DC expressed CD8, with a major group showing levels as high as on T cells or thymocytes (Fig. 3), confirming our earlier findings [10]. Our recent irradiated thymus reconstitution studies indicate that the cells expressing lower levels of CD8 are early, less mature DC, so it is likely that all mouse thymic DC will eventually express high levels of CD8 (Wu and Shortman, unpublished). Although most mouse thymus DC were clearly positive for CD4 when a high-sensitivity detection system was used, as in Fig. 3, the level of expression was 30-fold lower than on T cells and so would be considered 'low CD4'. By contrast most human thymic DC expressed very high levels of CD4, even higher than on T cells or thymocytes (Fig. 3). Two different anti-CD4 mAb gave similar high staining. Human thymic DC did appear to express some CD8, although it was only a little above the non-specific binding background and was about 30-fold lower in fluorescence intensity than on T cells or thymocytes. At this stage we do not know if the low level of CD4 on mouse DC, and of CD8 on human DC, was produced by the DC themselves or was passively acquired from previously adherent thymocytes.

These differences in CD4 and CD8 expression appeared to represent a species difference, rather than reflecting a difference in physiological age between the young adult mice (6 week) and infant humans (usually <2 years). Identical results to Fig. 2 were obtained with thymuses from 4-week-old mice, and with one thymus sample from a 10-year-old child (data not shown). In addition, using our previous less efficient isolation procedure, thymus tissue from a 15-year-old child yielded CD4^{hi} DC.

The expression on DC of very high levels of a surface molecule such as CD4 or CD8 would normally suggest the molecule is physiologically important. However the marked species difference in expression between thymic DC of mouse and man now argues

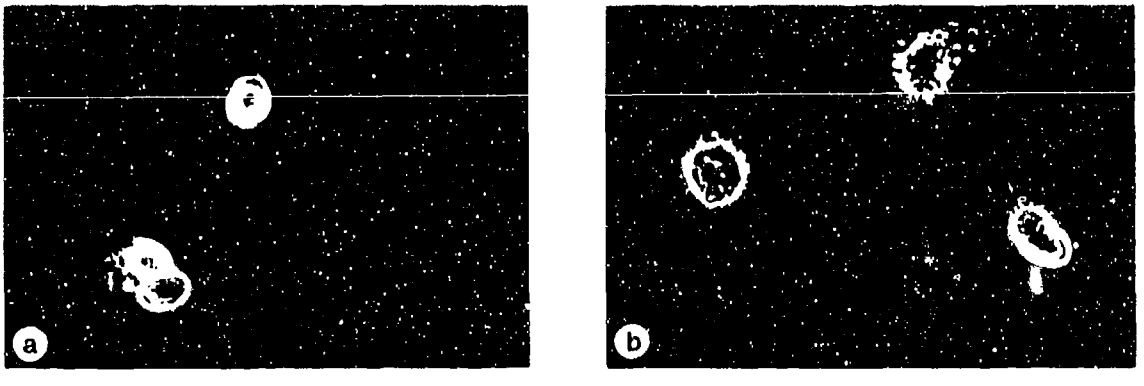

Fig. 2. The appearance of isolated thymic DC. The DC were isolated from thymus tissue then sorted as class II MHC^{hi} cells, as in Materials and Methods. They were then incubated for 1–5 h in simple culture medium, then placed in a microscope chamber and examined under phase contrast. Magnification is 1500-fold. Sample (a) is mouse thymus DC, sample (b) human thymus DC.

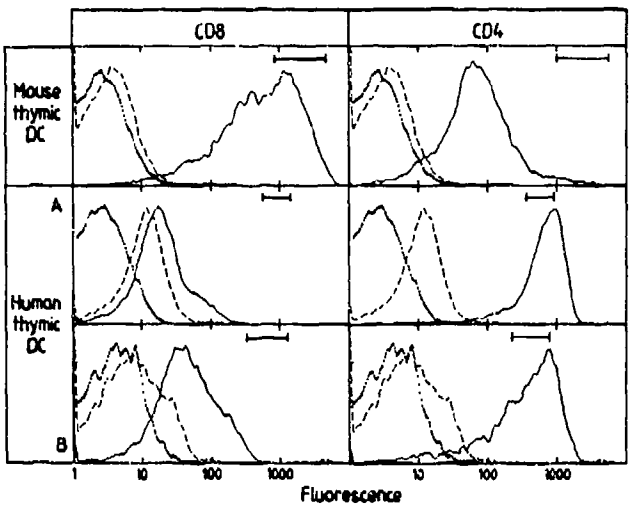

Fig. 3. The expression of CD4 and CD8 by mouse and human thymus DC. DC were isolated as described in Materials and Methods then stained in 2 fluorescent colors for class II MHC (green FITC fluorescence) and either CD4 or CD8 (red PE fluorescence). Samples were PI gated to exclude any dead or damaged cells, and gated to include cells with very high class II MHC expression together with high forward and side light scatter. The red fluorescence distribution of these gated DC is shown. The solid line is the fluorescence distribution of CD4 or CD8. The dotted line is the background autofluorescence, omitting only the PE-conjugated reagent. The dashed line is the fluorescence obtained with an isotype-matched irrelevant control mAb. For the human thymic DC, A and B represent separate preparations, sample B being stained after pre-incubation of the DC with mouse immunoglobulin to reduce the non-specific staining background. Such Ig blockage made no difference to the mouse thymic DC where non-specific binding was negligible. The bar insert represents the peak staining fluorescence obtained with whole thymocytes to provide a comparison with DC. Results are typical of 8 such experiments with mouse DC, 5 with human DC.

against some general functional role, unless the same basic function can be served by either CD4 or CD8. It could be argued that surface expression of these characteristic T cell surface components is no more than an accident, due to the development of DC in the same inductive environment as T cells, and that it serves no useful function at all. Or it could be argued that these molecules are serving as simple adhesion molecules via their interaction with class II or class I MHC, their role being a small part of a complex mosaic of multiple adhesion molecule interactions connecting thymic DC to both thymocytes and other thymic stromal elements. We have previously suggested a very specific role for CD8 on certain murine DC, namely to make a DC into 'veto' cell giving negative rather than positive signals to interacting T cells [9,10]. This proposal was based on a cell culture model where transfection of an antigen-presenting cell (APC) with CD8 caused apoptosis of interacting murine T cells [20]. Our hypothesis is now substantially weakened by the very low levels of CD8 now found on the corresponding DC from the human thymus, although it is conceivable that CD4 rather than CD8 on the APC could transmit such negative signals to human T cells.

Regardless of the normal physiological role of these molecules on DC, the finding that almost all human thymic DC express very high levels of CD4, a receptor for human immunodeficiency virus (HIV), may be of significance in the etiology of AIDS. The role of bone marrow-derived DC in the transmission of HIV to T cells, and the question of whether DC can themselves be infected with the virus, has been controversial [21,22]. Recently it has been shown that most human blood DC isolated without culture express CD4, although this is rapidly lost during culture in the presence of monocytes or macrophages [23]. These CD4-bearing blood DC are being considered as targets for HIV infection. Since virtually all human thymic DC express even higher levels of CD4, these DC must also be considered as likely targets of HIV-1 infection. Several recent studies where HIV-1 has been inoculated into fetal thymic implants in SCID-hu mice indicate efficient replication and progressive damage to the thymic microenvironment [24–26]. Monkey thymus is an early site of SIV infection, and this is followed by extensive thymus disruption [27]. The procedure we have used for isolating human thymic DC provides a ready source of $CD4^{hi}$ DC for experimental tests of their susceptibility to HIV infection.

Acknowledgements

We thank Dr. Frank Battye, Ralph Rossi and Robyn Muir for assistance with flow cytometry, and Dr. Barbara Moscicki for her help in developing techniques for disrupting thymic tissue. This work was supported by grants from the Australian Commonwealth AIDS Research Project Grant, and from the US Army Medical Research and Development Command.

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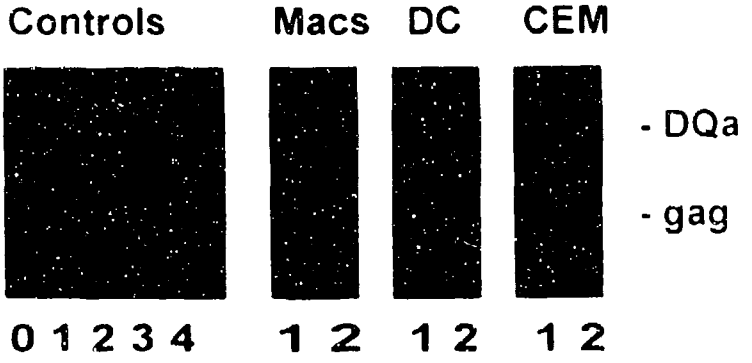
APPENDIX (d)

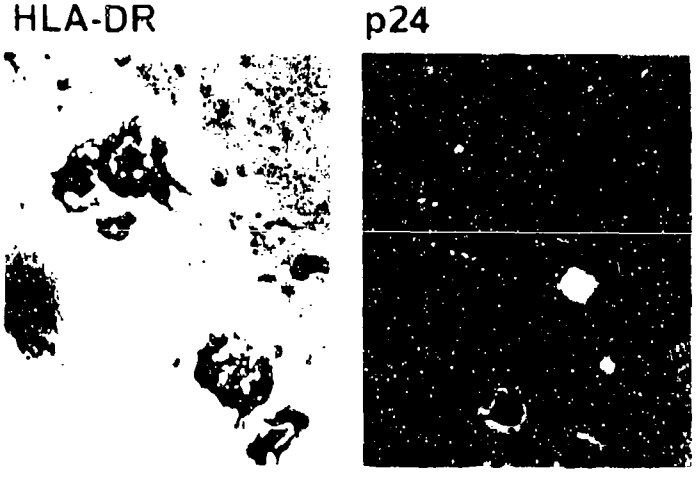
Evidence for infection of thymic dendritic cells with macrophage-trophic strains of HIV-1.

a. PCR analysis with gag-specific primers.



The controls are 8E5 cells containing known copy numbers of HIV-1. In the tests dendritic cells (DC) are compared with macrophages (Macs) and a lymphoid cell line (CEM). Lane 1 is from cells infected with IIIb (a T-cell trophic isolate), Lane 2 from cells infected with Ba-L (a macrophage trophic isolate).

b. Immunoperoxidase staining with antibody against p24



DC infected with Ba-L were cultured for 5 days, then stained for HLA-DR (to demonstrate DC survival) and p24 (to demonstrate HIV-1 infection).